ISPH-0533 PATENT

ANTISENSE MODULATION OF FOCAL ADHESION KINASE EXPRESSION

INTRODUCTION

This application is a continuation-in-part of the PCT Application No. PCT/US00/18999 filed July 13, 2000 which corresponds to U.S. Application No. 09/377,310 filed August 19, 1999 now issued U.S. Patent No. 6,133,031.

FIELD OF THE INVENTION

This invention relates to compositions and methods for modulating expression of the human focal adhesion kinase (FAK) gene, which encodes a signaling protein involved in growth factor response and cell migration and is implicated in disease. This invention is also directed to methods for inhibiting FAK-mediated signal transduction; these methods can be used diagnostically or therapeutically. Furthermore, this invention is directed to treatment of conditions associated with expression of the human FAK gene.

BACKGROUND OF THE INVENTION

Cell migration is fundamental to a variety of biological processes and can be induced by both integrin receptor-mediated signals (haptotaxis migration) and/or soluble growth factor-mediated signals (chemotaxis migration). Integrin receptor engagement activates focal adhesion kinase (FAK, also pp125FAK), a non-receptor protein-tyrosine kinase localized to cell substratum-extracellular matrix (ECM) contact sites that function as part of a cytoskeletal-associated network of signaling proteins (Schlaepfer, D.D., *et al.*, *Prog. Biophys. Mol. Biol.*, **1999**, *71*, 435-478). In adherent cells, FAK is often associated with integrins at focal adhesions (Schaller, M.D., *et al.*, *Proc. Natl. Acad. Sci. USA*, **1992**, *89*, 5192-5196). Numerous other signaling proteins, including other

protein tyrosine kinases are associated with FAK at these regions. Phosphorylation of FAK results in activation of the mitogen-activated protein kinase pathway. In addition, FAK regulates activation of phosphatidylinositol 3'-kinase which may serve to prevent apoptosis. FAK has also been shown to be required for internalization of bacteria mediated by invasin (Alrutz, M.A. and Isberg, R.R., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 13658-13663).

Normal cells typically require anchorage to the extracellular matrix in order to grow. When these cells are removed from the extracellular matrix, they undergo apoptosis. Transformed cells, on the other hand, can grow under anchorage-independent conditions, providing them a growth advantage and the ability to be removed from their normal cellular environment.

Overexpression of FAK is involved in cancer progression. High levels of FAK correlates with invasiveness and metastatic potential in colon tumors (Weiner, T.M., *et al.*, *Lancet*, **1993**, *342*, 1024-1025), breast tumors (Owens, L.V., *et al.*, *Cancer Res.*, **1995**, *55*, 2752-2755), and oral cancers (Kornberg, L.J., *Head Neck*, **1998**, *20*, 634-639).

FAK's role in cell migration has led to the speculation that it may be relevant in other diseases such as embryonic development disfunctions and angiogenic disorders (Kornberg, L.J., *Head Neck*, **1998**, *20*, 634-639).

There is a lack of specific inhibitors of FAK. Antisense approaches have been a means by which the function of FAK has been investigated. Lou, J. *et al.* (*J. Orthopaedic Res.*, **1997**, *15*, 911-918) used an adenoviral based vector to express antisense FAK RNA to show that FAK is involved in wound healing in tendons. Another antisense FAK expression vector containing 400 bp of complementary sequence was used to study the interaction of type I collagen and ?2?1 integrin

(Takeuchi, Y., *et al.*, *J. Biol. Chem.*, **1997**, *272*, 29309-29316).

Antisense oligonucleotides have been used in several studies. Tanaka, S. *et al.* (*J. Cell. Biochem.*, **1995**, *58*, 424-435) disclose two antisense phosphorothioate oligonucleotides targeted to the start site of mouse FAK. Xu, L.-H., *et al.* (*Cell Growth Diff.*, **1996**, *7*, 413-418) disclose two antisense phosphorothioate oligonucleotides targeted within the coding region of human FAK. They also show that FAK antisense treatment could induce apoptosis in tumor cells. Sonoda, Y., *et al.* (*Biochem. Biophys. Res. Comm.*, **1997**, *241*, 769-774) also demonstrated a role for FAK in apoptosis using antisense phosphorothioate oligonucleotides targeted to the start site and within the coding region of human FAK. Shibata, K., *et al.* (*Cancer Res.*, **1998**, *58*, 900-903) disclose antisense phosphorothioate oligonucleotides targeted to the start site and coding region of human FAK. Narase, K., *et al.* (*Oncogene*, **1998**, *17*, 455-463) disclose an antisense phosphorothioate oligonucleotide targeted to the start site of human FAK.

There remains a long-felt need for improved compositions and methods for inhibiting FAK gene expression.

SUMMARY OF THE INVENTION

The present invention provides antisense compounds which are targeted to nucleic acids encoding focal adhesion kinase expression (FAK) and are capable of modulating FAK mediated signaling. The present invention also provides chimeric oligonucleotides targeted to nucleic acids encoding human FAK. The antisense compounds of the invention are believed to be useful both diagnostically and therapeutically, and are believed to be particularly useful in the methods of the present invention.

The present invention also comprises methods of modulating FAK mediated signaling, in cells and tissues, using

the antisense compounds of the invention. Methods of inhibiting FAK expression are provided; these methods are believed to be useful both therapeutically and diagnostically. These methods are also useful as tools, for example, for detecting and determining the role of FAK in various cell functions and physiological processes and conditions and for diagnosing conditions associated with expression of FAK.

The present invention also comprises methods for diagnosing and treating cancers, including those of the colon, breast and mouth. These methods are believed to be useful, for example, in diagnosing FAK-associated disease progression. These methods employ the antisense compounds of the invention. These methods are believed to be useful both therapeutically, including prophylactically, and as clinical research and diagnostic tools.

DETAILED DESCRIPTION OF THE INVENTION

FAK plays important roles in integrin-mediated signal transduction. Overexpression of FAK is associated with tumor progression and metastatic potential. As such, this protein represents an attractive target for treatment of such diseases. In particular, modulation of the expression of FAK may be useful for the treatment of diseases such as colon cancer, breast cancer and cancer of the mouth.

The present invention employs antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding FAK, ultimately modulating the amount of FAK produced. This is accomplished by providing oligonucleotides which specifically hybridize with nucleic acids, preferably mRNA, encoding FAK.

This relationship between an antisense compound such as an oligonucleotide and its complementary nucleic acid target, to which it hybridizes, is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of this invention, is a

multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the targets are nucleic acids encoding FAK; in other words, a gene encoding FAK, or mRNA expressed from the FAK gene. mRNA which encodes FAK is presently the preferred target. The targeting process also includes determination of a site or sites within the nucleic acid sequence for the antisense interaction to occur such that modulation of gene expression will result.

In accordance with this invention, persons of ordinary skill in the art will understand that messenger RNA includes not only the information to encode a protein using the three letter genetic code, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region and intron/exon junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the informational ribonucleotides. The oligonucleotide may therefore be specifically hybridizable with a transcription initiation site region, a translation initiation codon region, a 5' cap region, an intron/exon junction, coding sequences, a translation termination codon region or sequences in the 5'- or 3'-untranslated region. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation

initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding FAK, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region," "AUG region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. This region is a preferred target region. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. This region is a preferred target region. The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other preferred target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site

and the translation initiation codon of an mRNA or corresponding nucleotides on the gene and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns", which are excised from a pre-mRNA transcript to yield one or more mature mRNA. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., exon-exon or intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. Targeting particular exons in alternatively spliced mRNAs may also be preferred. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired modulation.

"Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them.

"Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide.

It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment or, in the case of *in vitro* assays, under conditions in which the assays are conducted.

Hybridization of antisense oligonucleotides with mRNA interferes with one or more of the normal functions of mRNA. The functions of mRNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in by the RNA. Binding of specific protein(s) to the RNA may also be interfered with by antisense oligonucleotide hybridization to the RNA.

The overall effect of interference with mRNA function is modulation of expression of FAK. In the context of this invention "modulation" means either inhibition or stimulation; i.e., either a decrease or increase in expression. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression, or reverse transcriptase PCR, as taught in the examples of the instant application or by Western blot or ELISA assay of protein expression, or by an immunoprecipitation assay of protein expression. Effects on cell proliferation or tumor cell growth can also be measured, as taught in the examples of the instant application. Inhibition is presently preferred.

The oligonucleotides of this invention can be used in diagnostics, therapeutics, prophylaxis, and as research reagents and in kits. Since the oligonucleotides of this invention hybridize to nucleic acids encoding FAK, sandwich, colorimetric and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of oligonucleotide with the FAK genes or mRNA can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of FAK may also be prepared.

The present invention is also suitable for diagnosing abnormal inflammatory states or certain cancers in tissue or other samples from patients suspected of having an autoimmune or inflammatory disease such as hepatitis or cancers such as those of the colon, liver or lung, and lymphomas. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. In the context of this invention, to "contact" tissues or cells with an oligonucleotide or

oligonucleotides means to add the oligonucleotide(s), usually in a liquid carrier, to a cell suspension or tissue sample, either *in vitro* or *ex vivo*, or to administer the oligonucleotide(s) to cells or tissues within an animal.

The oligonucleotides of this invention may also be used for research purposes. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations and in other methodologies which may be appreciated by persons of ordinary skill in the art.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

The antisense compounds in accordance with this invention preferably comprise from about 5 to about 50 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2=, 3= or 5= hydroxyl moiety of the sugar. In

forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3= to 5= phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotri-esters, methyl and other alkyl phosphonates including 3=-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3=-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thiono-alkylphosphonates, thionoalkylphosphotriesters, and borano-phosphates having normal 3=-5= linkages, 2=-5= linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3=-5= to 5=-3= or 2=-5= to 5=-2=. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages

include, but are not limited to, U.S. Patents 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patents 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have

excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patents 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen *et al.* (*Science*, **1991**, *254*, 1497-1500).

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-alkyl-O-alkyl, O-, S-, or N-alkenyl, or O-, S- or N-alkynyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_2ON(CH_3)_2$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2= position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2,

heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, poly-alkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta* **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, and 2'-dimethylamino-ethoxyethoxy (2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_2)_2$.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2=-5= linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,0531 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and

natural nucleobases such as 5-methylcytosine (5-me-C or m5c), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. patent 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering* **1990**, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, those disclosed by Englisch *et al.* (*Angewandte Chemie, International Edition* **1991**, *30*, 613-722), and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications* **1993**, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications* **1993**, CRC Press, Boca Raton, pages 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. patent 3,687,808, as well as U.S. patents 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6553-6556), cholic acid (Manoharan *et al.*, *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1053-1059), a thioether, e.g., hexyl-*S*-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.* **1992**, *660*, 306-309; Manoharan *et al.*, *Bioorg. Med. Chem. Let.* **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.* **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J.* **1991**, *10*, 1111-1118; Kabanov *et al.*, *FEBS Lett.* **1990**, *259*, 327-330; Svinarchuk *et al.*, *Biochimie* **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.* **1995**, *36*, 3651-3654; Shea *et al.*, *Nucl. Acids Res.* **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides* **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.* **1995**, *36*, 3651-3654), a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta* **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol

moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.* **1996**, *277*, 923-937).

Representative U.S. patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. patents 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis

and, if necessary, associated nucleic acid hybridization techniques known in the art. This RNAse H-mediated cleavage of the RNA target is distinct from the use of ribozymes to cleave nucleic acids. Ribozymes are not comprehended by the present invention.

Examples of chimeric oligonucleotides include but are not limited to "gapmers," in which three distinct regions are present, normally with a central region flanked by two regions which are chemically equivalent to each other but distinct from the gap. A preferred example of a gapmer is an oligonucleotide in which a central portion (the "gap") of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, while the flanking portions (the 5' and 3' "wings") are modified to have greater affinity for the target RNA molecule but are unable to support nuclease activity (e.g., fluoro- or 2'-O-methoxyethyl-substituted). Chimeric oligonucleotides are not limited to those with modifications on the sugar, but may also include oligonucleosides or oligonucleotides with modified backbones, e.g., with regions of phosphorothioate (P=S) and phosphodiester (P=O) backbone linkages or with regions of MMI and P=S backbone linkages. Other chimeras include "wingmers," also known in the art as "hemimers," that is, oligonucleotides with two distinct regions. In a preferred example of a wingmer, the 5' portion of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl- substituted), or vice-versa. In one embodiment, the oligonucleotides of the present invention contain a 2'-O-methoxyethyl (2'-O-$CH_2CH_2OCH_3$) modification on the sugar moiety of at least one nucleotide. This modification has been shown to increase both affinity of the oligonucleotide for its target and nuclease resistance of

the oligonucleotide. According to the invention, one, a plurality, or all of the nucleotide subunits of the oligonucleotides of the invention may bear a 2'-O-methoxyethyl ($-O-CH_2CH_2OCH_3$) modification. Oligonucleotides comprising a plurality of nucleotide subunits having a 2'-O-methoxyethyl modification can have such a modification on any of the nucleotide subunits within the oligonucleotide, and may be chimeric oligonucleotides. Aside from or in addition to 2'-O-methoxyethyl modifications, oligonucleotides containing other modifications which enhance antisense efficacy, potency or target affinity are also preferred. Chimeric oligonucleotides comprising one or more such modifications are presently preferred.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-methoxyethyl oligonucleotides (Martin, P., *Helv. Chim. Acta* **1995**, *78*, 486-504). It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling, VA) to synthesize fluorescently labeled, biotinylated or other conjugated oligonucleotides.

The antisense compounds of the present invention include bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs. This is intended to encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to

an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of the nucleic acids of the invention and prodrugs of such nucleic acids. Pharmaceutically acceptable salts are physiologically and pharmaceutically acceptable salts of the nucleic acids of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto (see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.* **1977**, *66*, 1-19).

For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, *etc.*; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a Aprodrug form. The term Aprodrug indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE

[(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993.

For therapeutic or prophylactic treatment, oligonucleotides are administered in accordance with this invention. Oligonucleotide compounds of the invention may be formulated in a pharmaceutical composition, which may include pharmaceutically acceptable carriers, thickeners, diluents, buffers, preservatives, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients and the like in addition to the oligonucleotide. Such compositions and formulations are comprehended by the present invention.

Pharmaceutical compositions comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, *8*, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* **1990**, *7*, 1-33). One or more penetration enhancers from one or more of these broad categories may be included.

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (*i.e.*, oleate, laurate, caprate,

myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* **1990**, 7, 1; El-Hariri *et al.*, *J. Pharm. Pharmacol.* **1992** *44*, 651-654).

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, **1996**, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations.

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)[Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems* **1990**, 7, 1-33; Buur *et al.*, *J. Control Rel.* **1990**, *14*, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi *et al.*, *J. Pharm. Phamacol.* **1988**, *40*, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.* **1987**, *39*, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor.

In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinyl-pyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose,

pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrates (*e.g.*, starch, sodium starch glycolate, *etc.*); or wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. patents 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, *e.g.*, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the oligonucleotides of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of

liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layers made up of lipids arranged in a bilayer configuration (see, generally, Chonn *et al.*, *Current Op. Biotech.* **1995**, *6*, 698-708).

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, epidermal, and transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. In some cases it may be more effective to treat a patient with an oligonucleotide of the invention in conjunction with other traditional therapeutic modalities in order to increase the efficacy of a treatment regimen. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic,

palliative and prophylactic modalities. For example, a patient may be treated with conventional chemotherapeutic agents, particularly those used for tumor and cancer treatment. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide, trimetrexate, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp. 1206-1228, Berkow *et al.*, eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide).

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body

of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective *in vitro* and in *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

EXAMPLE 1: Synthesis of Oligonucleotides

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. ß-cyanoethyldiisopropyl-phosphoramidites are purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of ^{3}H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step. Cytosines may be 5-methyl cytosines.

(5-methyl deoxycytidine phosphoramidites available from Glen Research, Sterling, VA or Amersham Pharmacia Biotech, Piscataway, NJ)

2'-methoxy oligonucleotides are synthesized using 2'-methoxy ß-cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham, MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base is increased to 360 seconds. Other 2'-alkoxy oligonucleotides are synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2'-fluoro oligonucleotides are synthesized as described in Kawasaki *et al.* (*J. Med. Chem.* **1993**, *36*, 831-841). Briefly, the protected nucleoside N^6-benzoyl-2'-deoxy-2'-fluoroadenosine is synthesized utilizing commercially available 9-ß-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-a-fluoro atom is introduced by a S_N2-displacement of a 2'-ß-O-trifyl group. Thus N^6-benzoyl-9-ß-D-arabinofuranosyladenine is selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N^6-benzoyl groups is accomplished using standard methodologies and standard methods are used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine is accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-ß-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group is followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation is followed by treatment of the crude product with fluoride, then

deprotection of the THP groups. Standard methodologies are used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

Synthesis of 2'-deoxy-2'-fluorouridine is accomplished by the modification of a known procedure in which 2, 2'-anhydro-1-ß-D-arabinofuranosyluracil is treated with 70% hydrogen fluoride-pyridine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-deoxy-2'-fluorocytidine is synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N^4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-(2-methoxyethyl)-modified amidites were synthesized according to Martin, P. (*Helv. Chim. Acta* **1995**, *78*, 486-506). For ease of synthesis, the last nucleotide may be a deoxynucleotide. 2'-O-$CH_2CH_2OCH_3$-cytosines may be 5-methyl cytosines.

Synthesis of 5-Methyl cytosine monomers:

2,2'-Anhydro[1-(ß-D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/-Hexane/Acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product.

Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-uridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5?C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the

reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure

product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods (Sanghvi et al., *Nucl. Acids Res.* **1993**, *21*, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2=-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In

order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-

butyldiphenylsilyl-5-methyluridine as white foam (21.819, 86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 hr the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-*O*-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1eg.) was added and the mixture for 1 hr. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 hr, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na_2SO_4, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at

room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

<u>2'-O-(dimethylaminooxyethyl)-5-methyluridine</u>

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

<u>5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine</u>

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the

residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

<u>5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]</u>

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

Oligonucleotides having methylene(methylimino) (MMI) backbones are synthesized according to U.S. patent 5,378,825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. For ease of synthesis, various nucleoside dimers containing MMI linkages are synthesized and incorporated into oligonucleotides. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker *et al.* (*Acc. Chem. Res.* **1995**, *28*, 366-374). The amide moiety is readily accessible by simple

and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. patent 5,034,506 (Summerton and Weller).

Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen *et al.* (*Science* **1991**, *254*, 1497-1500).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels or capillary gel electrophoresis and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang *et al.* (*J. Biol. Chem.* **1991**, *266*, 18162). Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Alternatively, oligonucleotides are synthesized in 96 well plate format via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages are afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages are generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-di-isopropyl phosphoramidites are purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster

City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per published methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

EXAMPLE 2: Human FAK Oligonucleotide Sequences

Antisense oligonucleotides were designed to target human FAK. Target sequence data are from the focal adhesion kinase (FAK) cDNA sequence published by Whitney, G.S., *et al.* (*DNA Cell Biol.*, **1993**, *12*, 823-830); Genbank accession number L13616, provided herein as SEQ ID NO: 1. One set of oligonucleotides were synthesized as chimeric oligonucleotides ("gapmers"), 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All 2'-MOE cytosines were 5-methyl-cytosines. These oligonucleotide sequences are shown in Table 1. An identical set of sequences were prepared as fully phosphorothioated oligodeoxynucleotides. These are shown in Table 2. An additional set of oligonucleotides were synthesized as chimeric oligonucleotides ("gapmers"), 15 nucleotides in length, composed of a central "gap" region consisting of five 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings."

The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All 2'-MOE cytosines were 5-methyl-cytosines. These oligonucleotide sequences are shown in Table 3. An identical set of sequences were prepared as fully phosphorothioated oligodeoxynucleotides. These are shown in Table 4.

Human A549 lung carcinoma cells (American Type Culture Collection, Manassas, VA) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), non-essential amino acids for MEM, sodium pyruvate (1 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml). All cell culture reagents were obtained from Life Technologies (Rockville, MD).

The cells were washed once with OPTIMEM™ (Life Technologies, Rockville, MD), then transfected with 400 nM oligonucleotide and 12 mg/ml LIPOFECTIN® (Life Technologies, Rockville, MD), a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in membrane filtered water. The cells were incubated with oligonucleotide for four hours, after which the media was replaced with fresh media and the cells incubated for another 20 hours.

Total cellular RNA was isolated using an ATLAS™ Pure RNA isolation kit (Clontech, Palo Alto, CA). RNA was then separated on a 1.2% agarose-formaldehyde gel, transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech, Arlington Heights, IL), a positively charged nylon membrane. Immobilized RNA was cross-linked by exposure to UV light. Membranes were probed with either FAK or glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probes. The probes were labeled by random primer using the PRIME-A-GENE[7] Labeling System, Promega, Madison, WI) and hybridized to the membranes. mRNA signals were quantitated by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Results of an initial screen of the FAK antisense oligonucleotides are shown in Tables 5 (20 mers) and 6 (15 mers). Oligonucleotides 15392 (SEQ ID NO. 3), 15394 (SEQ ID NO. 4), 15397 (SEQ ID NO. 6), 15399 (SEQ ID NO. 7), 15401 (SEQ ID NO. 8), 15403 (SEQ ID NO. 9), 15405 (SEQ ID NO. 10), 15407 (SEQ ID NO. 11), 15409 (SEQ ID NO. 12), 15413 (SEQ ID NO. 14), 15415 (SEQ ID NO. 15), 15458 (SEQ ID NO. 16), 15460 (SEQ ID NO. 17), 15421 (SEQ ID NO. 18), 15425 (SEQ ID NO. 20), 15393 (SEQ ID NO. 23), 15406 (SEQ ID NO. 30), 15408 (SEQ ID NO. 31) and 15412 (SEQ ID NO. 33) resulted in about 50% or greater inhibition of FAK mRNA expression in this assay. Oligonucleotides 15401 (SEQ ID NO. 8), 15403 (SEQ ID NO. 9), 15409 (SEQ ID NO. 12), 15413 (SEQ ID NO. 14), 15415 (SEQ ID NO. 15), and 15421 (SEQ ID NO. 18) resulted in about 80% or greater inhibition of FAK mRNA expression.

TABLE 1: Nucleotide Sequences of Human FAK Chimeric (deoxy gapped) 20 mer Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
15392	**CCGCG**GGCTCACAGT**GGTCG**	3	0001-0020	5'-UTR
15394	**GGCGC**CGTGAAGCGA**AGGCA**	4	0078-0097	5'-UTR
15395	**CAGTT**CTGCTCGGAC**CGCGG**	5	0101-0120	5'-UTR
15397	**GAAAC**TGCAGAAGGC**ACTGA**	6	0150-0169	5'-UTR
15399	**TTCTC**CCTTCCGTTA**TTCTT**	7	0183-0202	5'-UTR
15401	**CTAGA**TGCTAGGTAT**CTGTC**	8	0206-0225	5'-UTR
15403	**TTTTG**CTAGATGCTA**GGTAT**	9	0211-0230	5'-UTR
15405	**GGTAA**GCAGCTGCCA**TTATT**	10	0229-0248	start
15407	**AGTAC**CCAGGTGAGT**CTTAG**	11	0285-0304	coding
15409	**CCTGA**CATCAGTAGC**ATCTC**	12	0408-0427	coding
15411	**GTTGG**CTTATCTTCA**GTAAA**	13	0641-0660	coding
15413	**GGTTA**GGGATGGTGC**CGTCA**	14	1218-1237	coding

15415	**TGTTG**GTTTCCAATC**GGACC**	15	2789-2808	coding
15417	**CTAGG**GGAGGCTCAG**TGTGG**	16	3383-3402	stop
15419	**ATTCC**TCGCTGCTGG**TGGAA**	17	3444-3463	3'-UTR
15421	**TTTCA**ACCAGATGGT**CATTC**	18	3510-3529	3'-UTR
15423	**TTCTG**AATATCATGA**TTGAA**	19	3590-3609	3'-UTR
15425	**CATGA**TGCTTAAAAG**CTTAC**	20	3658-3677	3'-UTR
15427	**AATGT**GAACATAAAT**TGTTC**	21	3680-3699	3'-UTR
15429	**AAGGT**AGTTTAGGAA**TTAAG**	22	3738-3757	3'-UTR

[1] Emboldened residues are 2'-methoxyethoxy residues, 2'-methoxyethoxy cytosine residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

[2] Coordinates from Genbank Accession No. L13616, locus name "HUMFAKX", SEQ ID NO. 1.

TABLE 2: Nucleotide Sequences of Human FAK 20 mer Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
15432	CCGCGGGCTCACAGTGGTCG	3	0001-0020	5'-UTR
15434	GGCGCCGTGAAGCGAAGGCA	4	0078-0097	5'-UTR
15436	CAGTTCTGCTCGGACCGCGG	5	0101-0120	5'-UTR
15438	GAAACTGCAGAAGGCACTGA	6	0150-0169	5'-UTR
15440	TTCTCCCTTCCGTTATTCTT	7	0183-0202	5'-UTR
15442	CTAGATGCTAGGTATCTGTC	8	0206-0225	5'-UTR
15444	TTTTGCTAGATGCTAGGTAT	9	0211-0230	5'-UTR
15446	GGTAAGCAGCTGCCATTATT	10	0229-0248	start
15448	AGTACCCAGGTGAGTCTTAG	11	0285-0304	coding
15450	CCTGACATCAGTAGCATCTC	12	0408-0427	coding
15452	GTTGGCTTATCTTCAGTAAA	13	0641-0660	coding
15454	GGTTAGGGATGGTGCCGTCA	14	1218-1237	coding
15456	TGTTGGTTTCCAATCGGACC	15	2789-2808	coding
15458	CTAGGGGAGGCTCAGTGTGG	16	3383-3402	stop
15460	ATTCCTCGCTGCTGGTGGAA	17	3444-3463	3'-UTR

15462	TTTCAACCAGATGGTCATTC	18	3510-3529	3'-UTR
15464	TTCTGAATATCATGATTGAA	19	3590-3609	3'-UTR
15466	CATGATGCTTAAAAGCTTAC	20	3658-3677	3'-UTR
15468	AATGTGAACATAAATTGTTC	21	3680-3699	3'-UTR
15470	AAGGTAGTTTAGGAATTAAG	22	3738-3757	3'-UTR

[1] All linkages are phosphorothioate linkages.

[2] Coordinates from Genbank Accession No. L13616, locus name "HUMFAKX", SEQ ID NO. 1.

TABLE 3: Nucleotide Sequences of Human FAK Chimeric (deoxy gapped) 15 mer Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
15393	**GCGGG**CTCAC**AGTGG**	23	0004-0018	5'-UTR
15431	**CGCCG**TGAAG**CGAAG**	24	0081-0095	5'-UTR
15396	**GTTCT**GCTCG**GACCG**	25	0104-0118	5'-UTR
15398	**AACTG**CAGAA**GGCAC**	26	0153-0167	5'-UTR
15400	**CTCCC**TTCCG**TTATT**	27	0186-0200	5'-UTR
15402	**AGATG**CTAGG**TATCT**	28	0209-0223	5'-UTR
15404	**TTGCT**AGATG**CTAGG**	29	0214-0228	5'-UTR
15406	**TAAGC**AGCTG**CCATT**	30	0232-0246	start
15408	**TACCC**AGGTG**AGTCT**	31	0288-0302	coding
15410	**TGACA**TCAGT**AGCAT**	32	0411-0425	coding
15412	**TGGCT**TATCT**TCAGT**	33	0644-0658	coding
15414	**TTAGG**GATGG**TGCCG**	34	1221-1235	coding
15416	**TTGGT**TTCCA**ATCGG**	35	2792-2806	coding
15418	**AGGGG**AGGCT**CAGTG**	36	3386-3400	stop
15420	**TCCTC**GCTGC**TGGTG**	37	3447-3461	3'-UTR
15422	**TCAAC**CAGAT**GGTCA**	38	3513-3527	3'-UTR
15424	**CTGAA**TATCA**TGATT**	39	3593-3607	3'-UTR
15426	**TGATG**CTTAA**AAGCT**	40	3661-3675	3'-UTR
15428	**TGTGA**ACATA**AATTG**	41	3683-3697	3'-UTR

15430	**GGTAG**TTTAG**GAATT**	42	3741-3755	3'-UTR

[1] Emboldened residues are 2'-methoxyethoxy residues, 2'-methoxyethoxy cytosine residues are 5-methyl-cytosines; all linkages are phosphorothioate linkages.

[2] Coordinates from Genbank Accession No. L13616, locus name "HUMFAKX", SEQ ID NO. 1.

TABLE 4: Nucleotide Sequences of Human FAK 15 mer Phosphorothioate Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE[1] (5' -> 3')	SEQ ID NO:	TARGET GENE NUCLEOTIDE CO-ORDINATES[2]	GENE TARGET REGION
15433	GCGGGCTCACAGTGG	23	0004-0018	5'-UTR
15435	CGCCGTGAAGCGAAG	24	0081-0095	5'-UTR
15437	GTTCTGCTCGGACCG	25	0104-0118	5'-UTR
15439	AACTGCAGAAGGCAC	26	0153-0167	5'-UTR
15441	CTCCCTTCCGTTATT	27	0186-0200	5'-UTR
15443	AGATGCTAGGTATCT	28	0209-0223	5'-UTR
15445	TTGCTAGATGCTAGG	29	0214-0228	5'-UTR
15447	TAAGCAGCTGCCATT	30	0232-0246	start
15449	TACCCAGGTGAGTCT	31	0288-0302	coding
15451	TGACATCAGTAGCAT	32	0411-0425	coding
15453	TGGCTTATCTTCAGT	33	0644-0658	coding
15455	TTAGGGATGGTGCCG	34	1221-1235	coding
15457	TTGGTTTCCAATCGG	35	2792-2806	coding
15459	AGGGGAGGCTCAGTG	36	3386-3400	stop
15461	TCCTCGCTGCTGGTG	37	3447-3461	3'-UTR
15463	TCAACCAGATGGTCA	38	3513-3527	3'-UTR
15465	CTGAATATCATGATT	39	3593-3607	3'-UTR
15467	TGATGCTTAAAAGCT	40	3661-3675	3'-UTR
15469	TGTGAACATAAATTG	41	3683-3697	3'-UTR
15471	GGTAGTTTAGGAATT	42	3741-3755	3'-UTR

[1] All linkages are phosphorothioate linkages.

[2] Coordinates from Genbank Accession No. L13616, locus name "HUMFAKX", SEQ ID NO. 1.

TABLE 5

Inhibition of Human Fak mRNA expression in A549 Cells by FAK 20 mer Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
control	---	---	100%	0%
15392	3	5'-UTR	29%	71%
15432	3	5'-UTR	108%	---
15394	4	5'-UTR	30%	70%
15434	4	5'-UTR	147%	---
15395	5	5'-UTR	57%	43%
15436	5	5'-UTR	88%	12%
15397	6	5'-UTR	31%	69%
15438	6	5'-UTR	64%	36%
15399	7	5'-UTR	48%	52%
15440	7	5'-UTR	92%	8%
15401	8	5'-UTR	17%	83%
15442	8	5'-UTR	63%	37%
15403	9	5'-UTR	17%	83%
15444	9	5'-UTR	111%	---
15405	10	start	46%	54%
15446	10	start	145%	---
15407	11	coding	36%	64%
15448	11	coding	90%	10%
15409	12	coding	13%	87%
15450	12	coding	149%	---
15411	13	coding	70%	30%
15452	13	coding	129%	---
15413	14	coding	22%	78%
15454	14	coding	82%	18%

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
15415	15	coding	20%	80%
15456	15	coding	88%	12%
15417	16	stop	56%	44%
15458	16	stop	39%	61%
15419	17	3'-UTR	55%	45%
15460	17	3'-UTR	42%	58%
15421	18	3'-UTR	20%	80%
15462	18	3'-UTR	60%	40%
15423	19	3'-UTR	55%	45%
15464	19	3'-UTR	97%	3%
15425	20	3'-UTR	51%	49%
15466	20	3'-UTR	74%	26%
15427	21	3'-UTR	67%	33%
15468	21	3'-UTR	131%	---
15429	22	3'-UTR	57%	43%
15470	22	3'-UTR	71%	29%

TABLE 6

Inhibition of Human Fak mRNA expression in A549 Cells by FAK 15 mer antisense oligonucleotides

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
control	---	---	100%	0%
15393	23	5'-UTR	40%	60%
15433	23	5'-UTR	160%	---
15431	24	5'-UTR	59%	41%
15435	24	5'-UTR	121%	---
15396	25	5'-UTR	76%	24%
15437	25	5'-UTR	123%	---
15398	26	5'-UTR	72%	28%

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
15439	26	5'-UTR	64%	36%
15400	27	5'-UTR	79%	21%
15441	27	5'-UTR	66%	34%
15402	28	5'-UTR	69%	31%
15443	28	5'-UTR	99%	1%
15404	29	5'-UTR	70%	30%
15445	29	5'-UTR	151%	---
15406	30	start	32%	68%
15447	30	start	69%	31%
15408	31	coding	35%	65%
15449	31	coding	89%	11%
15410	32	coding	67%	33%
15451	32	coding	142%	---
15412	33	coding	43%	57%
15453	33	coding	115%	---
15414	34	coding	64%	36%
15455	34	coding	59%	41%
15416	35	coding	69%	31%
15457	35	coding	121%	---
15418	36	stop	140%	---
15459	36	stop	72%	28%
15420	37	3'-UTR	158%	---
15461	37	3'-UTR	62%	38%
15422	38	3'-UTR	153%	---
15463	38	3'-UTR	91%	9%
15424	39	3'-UTR	207%	---
15465	39	3'-UTR	88%	12%
15426	40	3'-UTR	171%	---
15467	40	3'-UTR	105%	---
15428	41	3'-UTR	95%	5%
15469	41	3'-UTR	96%	4%
15430	42	3'-UTR	137%	---

ISIS No:	SEQ ID NO:	GENE TARGET REGION	% mRNA EXPRESSION	% mRNA INHIBITION
15471	42	3'-UTR	131%	---

EXAMPLE 3: Dose response of antisense phosphorothioate oligonucleotide effects on FAK levels in A549 cells

Several of the more active oligonucleotides were chosen for a dose response study. A549 cells were grown, treated and processed as described in Example 2, except the concentration of oligonucleotide was varied.

Results are shown in Table 7. Many oligonucleotides showed IC_{50}s of 50 nM or less and maximal inhibition seen was 95%.

TABLE 7

Dose Response of A549 cells to FAK Phosphorothioate Oligonucleotides

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
control	---	---	---	100.0%	---
15932	3	5'-UTR	50 nM	80.3%	19.7%
"	"	"	200 nM	41.6%	58.4%
"	"	"	400 nM	28.3%	71.7%
15393	23	5'-UTR	50 nM	116.6%	---
"	"	"	200 nM	87.8%	12.2%
"	"	"	400 nM	60.7%	39.3%
15401	8	5'-UTR	50 nM	31.9%	68.1%
"	"	"	200 nM	26.8%	73.2%
"	"	"	400 nM	20.4%	79.6%
15403	9	5'-UTR	50 nM	82.7%	17.3%
"	"	"	200 nM	27.8%	72.2%
"	"	"	400 nM	18.6%	81.4%

15406	30	start	50 nM	51.6%	48.4%
"	"	"	200 nM	40.5%	59.5%
"	"	"	400 nM	39.3%	60.7%
15408	31	coding	50 nM	47.7%	52.3%
"	"	"	200 nM	67.8%	32.2%
"	"	"	400 nM	53.2%	46.8%
15409	12	coding	50 nM	30.1%	69.9%
"	"	"	200 nM	29.7%	70.3%
"	"	"	400 nM	18.9%	81.1%
15413	14	coding	50 nM	45.6%	54.4%
"	"	"	200 nM	21.6%	78.4%
"	"	"	400 nM	20.6%	79.4%
15415	15	coding	50 nM	46.9%	53.1%
"	"	"	200 nM	18.0%	82.0%
"	"	"	400 nM	8.0%	92.0%
15421	18	3'-UTR	50 nM	25.0%	75.0%
"	"	"	200 nM	14.8%	85.2%
"	"	"	400 nM	5.0%	95.0%

A dose response experiment on protein levels was done with two oligonucleotides. A549 cells were grown and treated as described in Example 2 except the concentration was varied as shown in Table 3. The LIPOFECTIN[R] to oligonucleotide ratio was maintained at 3 mg/ml LIPOFECTIN[R] per 100 nM oligonucleotide. FAK protein levels were determined 48 hours after antisense treatment in whole cell lysates by anti-FAK blotting. Cells on 10cm plates were lysed with 0.5 ml modified RIPA lysis buffer, diluted with 0.5 ml HNTG buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), incubated with agarose beads, and cleared by centrifugation. Immunoprecipitations with a polyclonal FAK antibody (Salk Institute of Biological Studies, La Jolla, CA; additional FAK antibodies available from Upstate Biotechnology Incorporated, Lake Placid, NY) were performed for 4hr at 4°C, collected on protein A (Repligen, Cambridge, MA) or protein G-plus (Calbiochem) agarose beads, and the precipitated

protein complexes were washed at 4°C in Triton only lysis buffer (modified RIPA without sodium deoxycholate and SDS) followed by washing in HNTG buffer prior to direct analysis by SDS-PAGE. For immunoblotting, proteins were transferred to polyvinylidene fluoride membranes (Millipore) and incubated with a 1:1000 dilution of polyclonal antibody for 2 hr at room temperature. Bound primary antibody was visualized by enhanced chemiluminescent detection.

Results are shown in Table 8.

TABLE 8

Dose Response of A549 cells to FAK Phosphorothioate Oligonucleotides

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% protein Expression	% protein Inhibition
control	---	---	---	100%	---
15409	12	coding	25 nM	60%	40%
"	"	"	100 nM	57%	43%
"	"	"	200 nM	23%	77%
15421	18	3'-UTR	25 nM	73%	27%
"	"	"	100 nM	34%	66%
"	"	"	200 nM	24%	76%

EXAMPLE 4: Effect of FAK antisense phosphorothioate oligonucleotides on growth factor stimulated migration and invasion

Integrin-regulated focal adhesion kinase (FAK) is an important component of epidermal (EGF) and platelet-drived (PDGF) growth factor-induced motility of primary fibroblasts, smooth muscle, and adenocarcinoma cells. To measure the effect of FAK antisense oligonucleotides on cell migration, a modified Boyden chamber (Millipore, Bedford, MA) assay was used (Sieg, D.J., *et al.*, *J. Cell Sci.*, **1999**, *112*, 2677-2691).

Both membrane sides were coated with rat tail collagen (5 ?g/ml in PBS, Boehringer Mannheim) for 2 hr at 37°C, washed with PBS, and the chambers were placed into 24 well dishes containing migration media (0.5 ml DMEM containing 0.5% BSA) with or without human recombinant PDGF-BB, EGF, or basic-FGF (Calbiochem, San Diego, CA) at the indicated concentrations. Serum-starved A549 cells ($1x10^5$ cells in 0.3 ml migration media) were added to the upper chamber and after 3 hr at 37°C, the cells on the membrane upper surface were removed by a cotton tip applicator, the migratory cells on the lower membrane surface were fixed, stained (0.1% crystal violet, 0.1 M borate pH 9.0, 2% EtOH), and the dye eluted for absorbance measurements at 600 nM. Individual experiments represent the average from three individual chambers. Background levels of cell migration (less than 5% of total) in the absence of chemotaxis stimuli (0.5% BSA only) were subtracted from all points.

Results are shown in Table 9. ISIS 17636 (SEQ ID NO. 43) is a five base mismatch control oligonucleotide for ISIS 15421 (SEQ ID NO. 18).

TABLE 9

Effect of FAK Antisense Phosphorothioate Oligonucleotides on EGF-Stimulated Cell Migration

ISIS #	SEQ ID NO:	ASO Gene Target	EGF (ng/ml)	A_{600}
control	---	---	2.5	0.74
15421	18	3'-UTR	"	0.26
17636	43	control	"	0.90
control	---	---	5.0	0.89
15421	18	3'-UTR	"	0.25
17636	43	control	"	0.77

FAK antisense oligonucleotides were tested in an *in vitro* invasion assay using an ~1mm MATRIGEL^R (Becton Dickinson, Franklin Lakes, NJ) basement membrane barrier (Albini, A., *Pathol. Oncol. Res.*, **1998**, *4*, 230-241). Migration chambers were coated with the indicated concentration of MATRIGEL^R, dried under laminar flow and then rehydrated with cold serum free DMEM for 90 min on an orbital shaker. A549 cells were grown and transfected as described in Example 2. Cells ($1x10^5$) were then placed onto the MATRIGEL^R coated membrane and allowed to invade through the MATRIGEL^R towards a 10% FBS chemoattractant for the indicated times. Cells that invaded through the MATRIGEL^R were visualized by crystal violet staining as detailed in the migration assay. The amount of MATRIGEL^R was varied in the assay to show that invasion was being measured and that the migration was not serum-induced.

Results are shown in Table 10.

TABLE 10

Effect of FAK Antisense Phosphorothioate Oligonucleotides on Tumor Cell Invasion

ISIS #	SEQ ID NO:	ASO Gene Target	MATRIGEL^R (μg/chamber)	Migration (A_{600})
control	---	---	0	8.3
15421	18	3'-UTR	"	2.8
17636	43	control	"	9.9
control	---	---	15	4.5
15421	18	3'-UTR	"	2.0
17636	43	control	"	4.3
control	---	---	26	1.6
15421	18	3'-UTR	"	0.7
17636	43	control	"	1.3

EXAMPLE 5: FAK antisense oligonucleotides in a retinal neovascularization model

FAK antisense oligonucleotides were tested in a rabbit model of retinal neovascularization (Kimura, H., *et al.*, *Invest. Opthalmol. Vis. Sci.*, **1995**, *36*, 2110-2119). In this model, growth factors are encapsulated and injected beneath the retina.

Eight male Dutch Belt rabbits and one male Black Satin/New Zealand White Cross rabbit were used in this study. ISIS 15409 (SEQ ID NO. 12) was administered intravitreally by injection, once prior to surgical implantation of the polymeric pellets and once during pellet implantation. Retinal neovascularization was monitored by indirect opthalmolscopy and documented by fundus photography. Retinal neovascularization was graded on a scale from 1 to 5, with one being normal and five showing retinal hemorrhaging and/or detachment. In animals injected with saline and the growth factor containing pellets, evidence of retinal neovascularization could be detected in the first week and retinal hemorrhaging began by the end of the third week. Animals receiving the antisense FAK oligonucleotide showed no evidence of retinal neovascularization over a four week period.

EXAMPLE 6: Effect of FAK antisense phosphorothioate oligonucleotide (ISIS 15421) alone and in combination with 5-Flurouracil on the viability of melanoma cell lines

Inhibition of FAK in tumor cell lines causes cell rounding, loss of adhesion, and apoptosis which suggests a role for these inhibitors in the treatment of metastatic conditions. In these studies, an antisense inhibitor of FAK was tested alone and in combination with the chemotherapeutic agent, 5-FU for its effects on melanoma cell line viability.

C8161 and BL human melanoma cell lines were treated with ISIS 15421 (SEQ. ID. NO 18) or a control oligonucleotide, ISIS 29848, a 20-mer random oligonucleotide (NNNNNNNNNNNNNNNNNNNN, wherein each N is a mixture of A, C, G and T; herein incorporated as SEQ ID NO: 44) using the lipofectin protocol described herein. Oligonucleotides were transfected for four hours at 300 nM in lipofectin reagent and 5-FU (200 μg/mL; SIGMA) was added after the incubation for 20 hours. Cell viability was determined by the MTT assay. Loss of adhesion and apoptosis were determined by cell counting and the TUNEL assay, respectively. FAK expression was assayed by Western blot, probing with the anti-FAK clone 4.47 antibody (Upstate Biotechnology, Lake Placid, NY).

In The BL melanoma cell line, treatment with ISIS 15421 resulted in a 23% reduction in cell viability compared to control ($p<0.0001$). Addition of 5-FU to the antisense treated cells resulted in a significant further reduction in cell viability (69%; $p<0.0001$) compared to treatment with ISIS 15421 or 5-FU alone (4.4% reduction; $p=0.15$) or the control oligonucleotide, ISIS 29848. Similar results were seen with the C8161 cell line.

In both cell lines, reduction in cell viability was accompanied by a proportional loss of cell adhesion and an increase in apoptosis. Western blots showed that treatment with ISIS 15421 resulted in a decrease of FAK protein expression. FAK protein levels were decreased in BL melanoma cells upon treatment with 5-FU alone and were undetectable upon treatment with the combination of ISIS 15421 and 5-FU. These studies suggest that ISIS 15421, in combination with the chemotherapeutic agent 5-FU, may be a useful in the treatment of melanoma.

EXAMPLE 7: Effect of FAK antisense phosphorothioate oligonucleotide (ISIS 15421) on human melanoma xenograft tumor growth in mice

Another model used to investigate the efficacy of antisense oligonucleotides on tumor growth involves the use of mice transplanted with human cancer cells or cell line tumors. In these experiments human C8161 melanoma tumor xenografts were transplanted onto the side of nude mice with sutures or surgical staples. Mice were treated with ISIS 15421 (SEQ ID NO. 18) or the control ISIS 29848 (SEQ ID NO. 44) over a 28 day treatment course.

At the end of the timecourse, mice were sacrificed and tumor volumes measured. Tumor volumes in the antisense treated mice were significantly smaller than tumor volumes in control-treated mice with no observation of toxicity to the mice. Additionally, one third of the control-treated mice had grossly evident intraperitoneal metastases, while none of the antisense-treated mice displayed such metastases. These studies suggest that antisense oligonucleotides represent potential chemotherapeutic agents in the treatment of melanoma and the prevention of tumor metastasis.